REPORT

Missense Mutations in *SLC26A8*, Encoding a Sperm-Specific Activator of CFTR, Are Associated with Human Asthenozoospermia

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The cystic fibrosis transmembrane conductance regulator (CFTR) is present in mature sperm and is required for sperm motility and capacitation. Both these processes are controlled by ions fluxes and are essential for fertilization. We have shown that SLC26A8, a sperm-specific member of the SLC26 family of anion exchangers, associates with the CFTR channel and strongly stimulates its activity. This suggests that the two proteins cooperate to regulate the anion fluxes required for correct sperm motility and capacitation. Here, we report on three heterozygous *SLC26A8* missense mutations identified in a cohort of 146 men presenting with asthenozoospermia: c.260G>A (p.Arg87Gln), c.2434G>A (p.Glu812Lys), and c.2860C>T (p.Arg954Cys). These mutations were not present in 121 controls matched for ethnicity, and statistical analysis on a control population of 8,600 individuals (from dbSNP and 1000 Genomes) showed them to be associated with asthenozoospermia with a power > 95%. By cotransfecting Chinese hamster ovary (CHO)-K1 cells with SLC26A8 variants and CFTR, we showed that the physical interaction between the two proteins was partly conserved but that the capacity to activate CFTR-dependent anion transport was completely abolished for all mutants. Biochemical studies revealed the presence of much smaller amounts of protein for all variants, but these amounts were restored to wild-type levels upon treatment with the proteasome inhibitor MG132. Immunocytochemistry also showed the amounts of SLC26A8 in sperm to be abnormally small in individuals carrying the mutations. These mutations might therefore impair formation of the SLC26A8-CFTR complex, principally by affecting SLC26A8 stability, consistent with an impairment of CFTR-dependent sperm-activation events in affected individuals.

Infertility is a major public health issue currently affecting an estimated 7%–12% of couples worldwide. Various sperm defects, including asthenozoospermia, an impairment of sperm motility, are responsible for male infertility. Asthenozoospermia is detected in more than 40% of infertile men and is often associated with lower levels of sperm production and morphological abnormalities (oligoasthenoteratozoospermia).[1] Despite its high prevalence, very little is known about the genetic abnormalities potentially underlying human asthenozoospermia.

Ion fluxes play an essential role in the control of sperm motility and capacitation, a maturation event that occurs in the female genital tract and is required for fertilization. In particular, calcium, chloride, and bicarbonate influxes induce an increase in intracellular cyclic AMP concentration, leading to the activation of protein kinase A and phosphorylation cascades required for sperm motility and capacitation.[2–6] We previously described SLC26A8, also known as testis anion transporter 1 (TAT1), as a male-germ-cell-specific member of the large family of anion transporters, solute-linked carrier 26 (SLC26).[7]

Homozygous deletion of *Slc26A8* in mice leads to male sterility as a result of a total lack of sperm motility, an impairment of sperm capacitation, and severe structural defects of the flagellum (midpiece disorganization, hairpin-like bending of the flagellum, and atrophy of the annulus).[8] In agreement with this phenotype, SLC26A8 is located in the equatorial segment of the head and in the annulus, a ring-shaped structure at the junction between the midpiece and the principal piece of the flagellum, in mature sperm.[8,9]

Most members of the SLC26 family transport monovalent and/or divalent anions (including sulfate, chloride, bicarbonate, iodide, and oxalate ions), and individual members of the *SLC26* family might be specifically or preferentially expressed in particular tissues.[10] In humans, mutations in *SLC26A2* (MIM 606718), *SLC26A3* (MIM 126650), *SLC26A4* (MIM 605646), and *SLC26A5* (MIM 604943) have been causally associated with diastrophic dysplasia (MIM 222600), chloride diarrhea (MIM 214700), Pendred syndrome (MIM 274600), and deafness (MIM 613865), respectively.[11]

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Table 1. Sperm Characteristics of Individuals with Heterozygous Sequence Variants of *SLC26A8*

	Individual			
	X1	X2	X3	Lower Reference Limit[a]
DNA mutation	c.260G>A	c.2434G>A	c.2860C>T	-
Protein alteration	p.Arg87Gln	p.Glu812Lys	p.Arg954Cys	-
Age (years)	44	42	31	-
Volume of ejaculate (ml)	1.3	3	2.1	1.5
pH	8.1	7.7	7.9	7.2
Concentration (10^6 sperm/ml)	252	410	10	15
Progressive motility (PR) (%)	25	30	15	32
Viability (%)	83	79	69	58
Typical forms (%)	16	38	1	4
Morphological defects (%)	flagellum (19)	flagellum (10)	flagellum (39) and head (98)	-

The following abbreviation is used: PR, percentage of progressive sperm.
[a]Values were compared with the lower reference limits established by the World Health Organization.[21]

SLC26 transporters (SLC26A3, SLC26A4, SLC26A5, SLC26A6, and SLC26A9), present in various epithelia, form complexes with the cystic fibrosis transmembrane conductance regulator (CFTR), the chloride and bicarbonate channel that is defective in cystic fibrosis (CF [MIM 219700]), and are able to regulate CFTR transport activity.[12–18] The CFTR channel has been detected in the equatorial segment of the head and in the flagellum (midpiece) of mature sperm and is required for sperm motility and capacitation.[19,20] We recently demonstrated that SLC26A8 (1) colocalizes with CFTR in mature sperm, (2) physically interacts with CFTR in vitro and in vivo, and (3) is a strong activator of CFTR. These observations implicate the SLC26A8-CFTR complex in the in vivo regulation of the chloride and bicarbonate fluxes required for sperm motility and capacitation.[9]

We therefore suspected that mutations of *SLC26A8* (MIM 608480) and *CFTR* (MIM 602421) would impair sperm motility and capacitation. The aim of this study was to search for *SLC26A8* mutations in men with asthenozoospermia and sperm with a low fertilizing capacity. The entire study was performed in accordance with ethical guidelines and was approved by the ethics evaluation committee of the Institut National de la Santé et de la Recherche Médicale (authorization number 01-013) and by the Comité de Protection des Personnes CPP Ile de France III (authorization number Sc-2748). Informed consent was obtained from all individuals included in the study.

We analyzed the 19 exons composing the coding sequence of *SLC26A8* by denaturating high-performance liquid chromatography and direct sequencing in a cohort of 146 men being treated for infertility at the Reproductive Medicine Department of Cochin Hospital. The criteria for inclusion in the study were asthenozoospermia, defined as a percentage of progressive sperm (PR) in the ejaculate of less than 32%, and normal vitality, defined as a percentage of viable sperm exceeding 58%, and were in line with the current values established by the World Health Organization.[21] Twenty-seven of the 146 men had severe asthenozoospermia with a PR < 15%, 67 had a PR between 20% and 25%, and 50 had a PR = 30%.

We identified seven asthenozoospermic individuals carrying heterozygous variants of the coding regions of *SLC26A8* (RefSeq accession number NM_052961.3); these variants were absent from the alleles of 121 controls matched for ethnicity. Only three of these sequence variants—c.260G>A (p.Arg87Gln), c.2434G>A (p.Glu812Lys), and c.2860C>T (p.Arg954Cys)—were deleterious in terms of their effects on functional cooperation between SLC26A8 and CFTR (see below); these three variants were studied in greater detail and are presented in this report.

The main semen parameters for individuals X1, X2, and X3, carrying the sequence variants c.260G>A (p.Arg87Gln), c.2434G>A (p.Glu812Lys), and c.2860C>T (p.Arg954Cys), respectively, are presented in Table 1. Two of these subjects (individuals X1 and X2) displayed moderate asthenozoospermia, and the third (individual X3) had more severe asthenozoospermia. Individual X1 had a low ejaculate volume and a frequency of sperm morphological abnormalities moderately higher than normal: in particular, coiled tails (19%) and an abnormally shaped posterior part of the sperm head (63%) were observed. Furthermore, the concentration of polymorphonuclear leukocytes exceeded 1×10^6/ml (LeucoScreen assay). All other semen parameters were normal in individual X2, although flagellum defects were observed in about 10% of the sperm. Individual X3 had a low sperm count and a very high frequency of combined morphological defects, including abnormal head shape (98%), midpiece abnormalities (27%), and flagellum defects (38%). No bacteria were detected in cultures of semen from these three individuals.

All three individuals (X1, X2, and X3) were consulting for primary infertility. Individual X1 is an only child

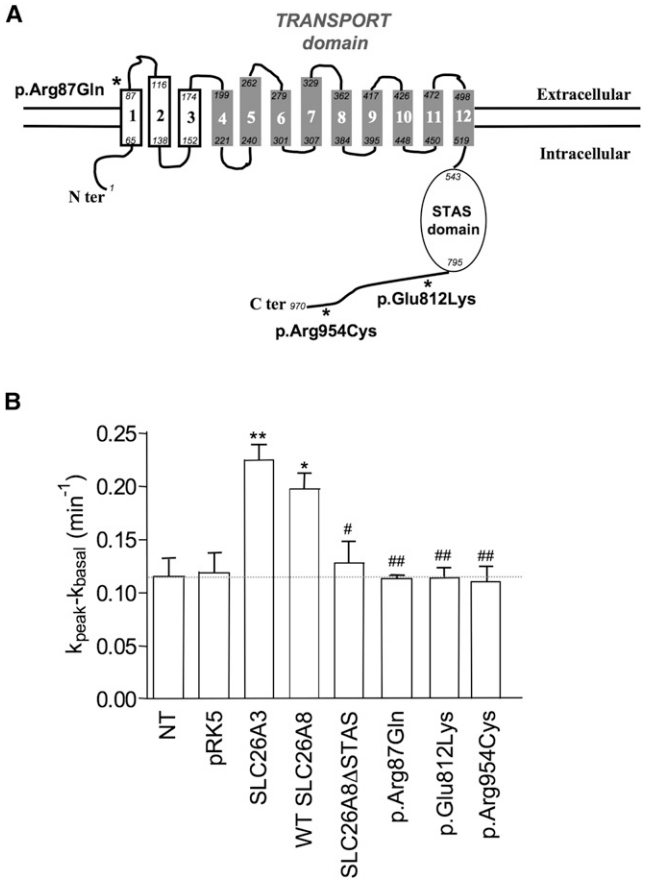

Figure 1. Distribution of SLC26A8 Sequence Variants and Their Effects on CFTR Stimulation

(A) SLC26A8 is composed of 12 transmembrane helices, including the transport domain, and a large cytoplasmic region that includes the regulatory STAS domain (amino acids 543–795). The p.Arg87Gln (c.260G>A) substitution maps to the junction between the first transmembrane domain and the extracellular loop of the protein; the p.Glu812Lys (c.2434G>A) and p.Arg954Cys (c.2860C>T) substitutions map to the C terminus.

(B) CFTR anion-transport activity was measured by iodide (^{125}I)-efflux experiments in CHO-K1 cells stably expressing the wild-type human CFTR (CHO-CFTR) and transiently transfected with the following expression vectors: pRK5 (empty vector), pCMV-SLC26A3, pRK5-SLC26A8, pRK5-SLC26A8ΔSTAS (a truncated SLC26A8 lacking the STAS domain), pRK5-p.Arg87Gln, pRK5-p.Glu812Lys, and pRK5-p.Arg954Cys. The experimental procedures have been described elsewhere.[22] Asterisks indicate that the mean significantly differs from that for pRK5 (t test p values = 0.0015 [SLC26A3] and 0.0068 [SLC26A8]). Number signs indicate that the mean significantly differs from that for wild-type (WT) SLC26A8 (t test p values = 0.0295 [SLC26A8Δ STAS], 0.0010 [p.Arg87Gln], 0.0020 [p.Glu812Lys], and 0.0049 [p.Arg954Cys]). The following abbreviation is used: NT, no transfection.

with an unknown father, and his mother has one brother with no children. Individual X2 has two sisters and four brothers, all of whom have children. Individual X3 was conceived after primary infertility of 10 years' duration; he is an only child, like his father, and his mother has one brother and one sister with children.

None of the members of the families of these three individuals were available for genetic study. For confirmation of the association between the variants (c.260G>A [p.Arg87Gln], c.2434G>A [p.Glu812Lys], and c.2860C>T [p.Arg954Cys]) and human asthenozoospermia, we therefore calculated the frequency of these alleles in large European control populations available from the National Center for Biotechnology Information website and the 1000 Genomes database (8,600 individuals) and used a Chi-square test to compare frequencies. For all three identified variants, the frequency of the mutant allele was higher (with a power > 95%) in the cohort of asthenozoospermic individuals than in the control groups (Table S1, available online). The sex and fertility status of the control individuals were unknown; the presence of infertile men potentially harboring *SLC26A8* mutations in the control population would increase the power of the analysis even further.

SLC26 family members have a common structure comprising a membranous sulfate transport domain with 12 putative transmembrane helices and a cytoplasmic regulatory domain, known as the STAS (sulfate transporter and antisigma factor antagonist) domain, at the C terminus.[10] The c.260G>A (p.Arg87Gln) mutation affects a residue located at the junction between the first transmembrane domain and the extracellular loop, and both the c.2434G>A (p.Glu812Lys) and c.2860C>T (p.Arg954Cys) mutations affect residues in the intracytoplasmic C-terminal region (Figure 1A).

We first assessed the possible pathogenicity of these sequence variants by in silico analysis with PolyPhen. The c.260G>A (p.Arg87Gln) variant (individual X1) was scored as "possibly damaging" (score 0.916), whereas variants c.2434G>A (p.Glu812Lys) (individual X2) and c.2860C>T (p.Arg954Cys) (individual X3) were both classified as "probably damaging" (scores 0.985 and 0.993, respectively). Next, we assessed the pathogenic effects of these variants experimentally by establishing eukaryotic expression vectors for each variant, and we then used these vectors for the transient transfection of Chinese hamster ovary (CHO-K1) cells; we used this model to determine the abundance and the properties of the variants in terms of their interaction and cooperation with the CFTR channel.

We performed iodide-efflux experiments by using a station for robotic flux-cell-based assays to assess the effects of the variants on CFTR activity.[22] We transiently transfected CHO-K1 cells stably expressing the CFTR channel with the *SLC26A8* wild-type or variant cDNA and induced CFTR activity with forskolin. As previously described, SLC26A8, like other SLC26 members (SLC26A3), strongly stimulated CFTR-associated iodide efflux.[9] The STAS domain mediates the interaction between several of the SLC26 members and CFTR and is required for CFTR stimulation.[12] We therefore used a truncated SLC26A8 lacking the STAS domain (SLC26A8ΔSTAS) as a negative control. The c.260G>A (p.Arg87Gln), c.2434G>A (p.Glu812Lys), and c.2860C>T (p.Arg954Cys) variants, like the protein encoded by the *SLC26A8ΔSTAS* construct, failed to stimulate CFTR (Figure 1B).

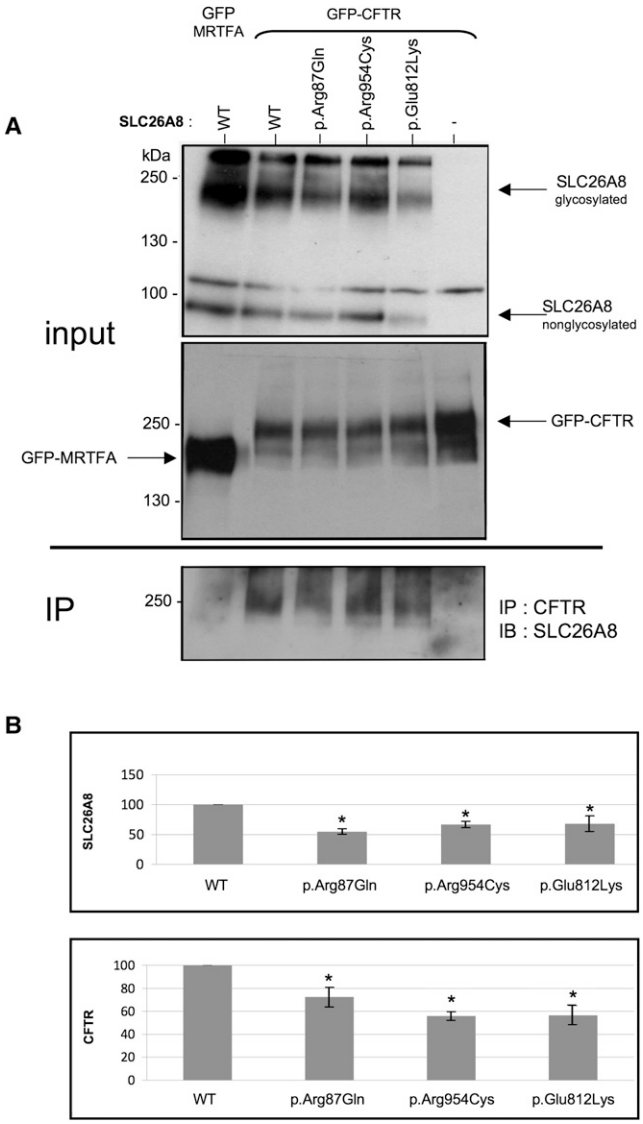

Figure 2. Protein Levels and Interaction between SLC26A8 Variants and the CFTR Channel

(A) The interaction between the p.Arg87Gln, p.Glu812Lys, and p.Arg954Cys variants and the CFTR channel in CHO-K1 cells was assessed by coimmunoprecipitation. Cells were transiently transfected with pRK5-SLC26A8, pRK5-p.Arg87Gln, pRK5-p.Glu812Lys, or pRK5-p.Arg954Cys and pGFP-CFTR or pGFP-MRTFA (negative control). A monoclonal CFTR antibody (M3A7; Millipore) was used for immunoprecipitation (IP), and a polyclonal SLC26A8 antibody (L2CL4) was used for immunoblotting (IB) of the precipitate, as previously described.[9]

(B) The amounts of SLC26A8 variants were determined after the transient transfection of CHO-K1 cells with pRK5-SLC26A8, pRK5-p.Arg87Gln, pRK5-p.Glu812Lys, or pRK5-p.Arg954Cys. Both glycosylated and nonglycosylated forms of SLC26A8 were assayed; β-tubulin was used as an internal loading control. The histogram shows the mean values of five experiments for the glycosylated form. Asterisks indicate that the mean significantly differs from that of the wild-type ($p < 0.05$) (t test p values = 0.0002 [p.Arg87Gln], 0.0013 [p.Arg954Cys], and 0.0333 [p.Glu812Lys]). The abundance of CFTR, when cotransfected with SLC26A8 variants, was analyzed in a similar manner. Bands A, B, and C of CFTR, corresponding to the nonglycosylated and glycosylated forms of CFTR, were studied. The histogram represents the mean of four experiments for CFTR band B. The asterisks indicate that the mean significantly differs from that of the wild-type ($p < 0.05$) (t test p values = 0.024 [p.Arg87Gln], 0.001 [p.Arg954Cys], and 0.007 [p.Glu812Lys]).

We then analyzed the interaction between the p.Arg87Gln, p.Glu812Lys, and p.Arg954Cys variants and CFTR in CHO-K1 cells by coimmunoprecipitation experiments. All three variants were found to be associated with CFTR, but the coimmunoprecipitation yield appeared to be slightly lower than that for the wild-type (Figure 2A).

We quantified the total amounts of the variants p.Arg87Gln, p.Glu812Lys, and p.Arg954Cys produced in the transfected cells. The altered proteins were significantly less abundant than the wild-type SLC26A8. The results of a representative immunoblotting experiment showing the loading controls are shown in Figure S1. Several of the extracellular residues of SLC26A8 are N-glycosylated.[7] We found that both nonglycosylated (not shown) and glycosylated forms of the variants were less abundant than the wild-type SLC26A8 forms: the amounts of p.Arg87Gln, p.Glu812Lys, and p.Arg954Cys were only 54%, 68%, and 66%, respectively, of that of the wild-type (n = 5; t test p values = 0.0002 [p.Arg87Gln], 0.0333 [p.Glu812Lys], and 0.0013 [p.Arg954Cys]) (Figure 2B).

Cells cotransfected with the c.260G>A (p.Arg87Gln), c.2434G>A (p.Glu812Lys), and c.2860C>T (p.Arg954Cys) variants and the wild-type CFTR also resulted in lower accumulation of CFTR than did control cells; the results of a representative immunoblotting experiment are shown in Figure S1. Like SLC26A8, CFTR is glycosylated, and three bands reflecting different maturation states of the protein were detected on immunoblots.[23] We quantified each band and found that the amounts of nonglycosylated forms of CFTR (CFTR bands A and B) were significantly smaller in cells cotransfected with the c.260G>A (p.Arg87Gln), c.2434G>A (p.Glu812Lys), or c.2860C>T (p.Arg954Cys) variant. The intensity of CFTR band B was 72%, 56%, and 55% of that in control cells when CFTR was cotransfected with c.260G>A (p.Arg87Gln), c.2434G>A (p.Glu812Lys), and c.2860C>T (p.Arg954Cys), respectively (n = 4; t test p values = 0.024, 0.007, and 0.001, respectively; Figure 2B). The abundance of the mature, fully glycosylated form of CFTR (band C) was also lower in cells in which it was cotransfected with the c.2434G>A (p.Glu812Lys) or c.2860C>T (p.Arg954Cys) variant (not shown).

These results suggest that the c.260G>A (p.Arg87Gln), c.2434G>A (p.Glu812Lys), and c.2860C>T (p.Arg954Cys) mutations decrease SLC26A8 stability and potentially affect the formation of the SLC26A8-CFTR complex.

We tested this hypothesis by quantifying the amounts of the variant proteins in cells treated with the peptide-aldehyde proteasome inhibitor MG132. After treatment with MG132, the abundances of all three altered proteins were found to be similar to that of the wild-type SLC26A8 (Figure 3; n = 4: t test p values = 0.014

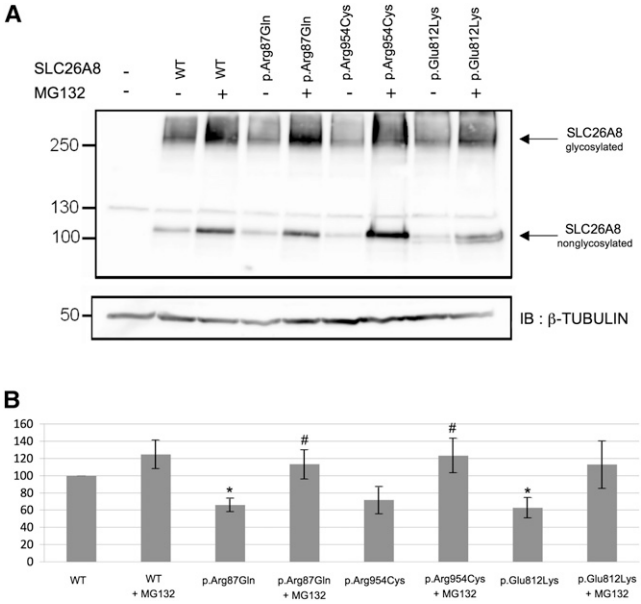

Figure 3. Levels of SLC26A8 Variants in Cells Treated with the Proteasome Inhibitor MG132

(A) Results of a representative immunoblot experiment after the transient transfection of CHO-K1 cells with pRK5-SLC26A8, pRK5-p.Arg87Gln, pRK5-p.Glu812Lys, or pRK5-p.Arg954Cys and treatment with 20 μM MG132 for 6 hr. A polyclonal SLC26A8 antibody (L2CL4) and a β-tubulin antibody were used for the immunoblotting (IB) analysis and as a loading control, respectively. (B) Quantification of the amounts of SLC26A8 variants after MG132 treatment. The histogram represents the mean of four experiments for the glycosylated form. Asterisks indicate that the mean significantly differs from that of the wild-type ($p < 0.05$) (t test p values = 0.022 [p.Arg87Gln], 0.040 [p.Glu812Lys], and 0.168 [p.Arg954Cys]). Number signs indicate that the mean significantly differs from that of the control (no MG132 treatment) ($p < 0.05$) (t test p values = 0.014 [p.Arg87Gln], 0.074 [p.Glu812Lys], and 0.015 [p.Arg954Cys]).

[p.Arg87Gln], 0.015 ([p.Arg954Cys], and 0.074 [p.Glu812Lys]). This suggests that the lower abundance of the variants than of the control protein results from their instability and proteasomal degradation.

We assessed the impact of the variants in vivo by the immunodetection of SLC26A8 in sperm preparations from individuals X2 (c.2434G>A [p.Glu812Lys]) and X3 (c.2860C>T [p.Arg954Cys]). Too little semen was available from individual X1 (c.260G>A [p.Arg87Gln]) for this test. We used an antibody recognizing both amino- and carboxy-terminal peptides from human SLC26A8.[9] In three independent experiments, SLC26A8 was detected at the annulus and in the equatorial segment of spermatozoa from both individuals and had a signal intensity lower than that of control sperm (Figure 4A). In addition, sperm from both individuals displayed morphological defects that were visible on light microscopy. We conducted a more detailed characterization of samples from individual X2 (c.2434G>A [p.Glu812Lys]): mitochondrial staining revealed an irregular organization of the midpiece, which was confirmed by electron microscopy (Figure S2). In most sperm from individual X3 (c.2860C>T [p.Arg954Cys]), SLC26A8 labeling, when present, was abnormally diffuse along the midpiece of the flagellum or absent from the equatorial segment of the head (Figure 4B). Such mislocalization in vivo would probably impair the formation of the SLC26A8-CFTR complex and might therefore potentially contribute to the observed sperm motility defects.

In conclusion, we report the identification and characterization of heterozygous *SLC26A8* missense mutations that abolish functional interaction with the CFTR channel and potentially contribute to subfertility in men by impairing sperm motility and capacitation. We show that the c.260G>A (p.Arg87Gln), c.2434G>A (p.Glu812Lys), and c.2860C>T (p.Arg954Cys) mutations impair the formation and stability of the SLC26A8-CFTR complex through their effects on SLC26A8 stability and abundance. These findings are consistent with the fact that smaller amounts of SLC26A8 lead to the presence of too few active SLC26A8-CFTR complexes in sperm.

Despite our best attempts, we were unable to obtain DNA from members of the families of these three individuals, and we therefore have no data about the inheritance of the mutations. All diseases associated with mutations in *SLC26* genes (*SLC26A2*, *SLC26A3*, *SLC26A4*, and *SLC26A5*) are autosomal recessive.[25] The mutations we identified in *SLC26A8* were found to be heterozygous in all individuals, suggesting a different mode of inheritance. This difference might be accounted for by the haploid state of the germ cells and the unequal sharing of material between them, despite their connection via intercellular bridges throughout spermatogenesis. These unique features of the sperm cell probably strengthen any phenotype resulting from monogenic heterozygous alterations and contribute to the heterogeneity of sperm phenotype in a given individual. Consistent with this, the individuals described here presented with mild asthenozoospermia and heterogeneous sperm phenotypes in terms of sperm motility and morphology.

Sperm from two of the individuals studied also displayed morphological abnormalities, such as midpiece disorganization and an incomplete annulus, similar to the defects observed in Slc26A8-null sperm in mice.[8] This phenotype is consistent with the dual function of SLC26A8, acting as a regulator of anion flux and a structural component of the annulus, the integrity of which is required for correct flagellum assembly.[8,24,26–28]

Overall, the clinical study presented here confirms the importance of functional cooperation between SLC26 family members and the CFTR channel. Consistent with this concept, a recent genome-wide association study identified several *SLC26A9* (MIM 608481) SNPs affecting the cystic fibrosis (CF) phenotype. Thus, although *CFTR* remains the only gene in which mutations cause CF, modulation of SLC26 activity might affect the CF phenotype in affected individuals.[29] Such cooperation between members of the SLC26 family and CFTR has now been described in several tissues, including the epithelia.

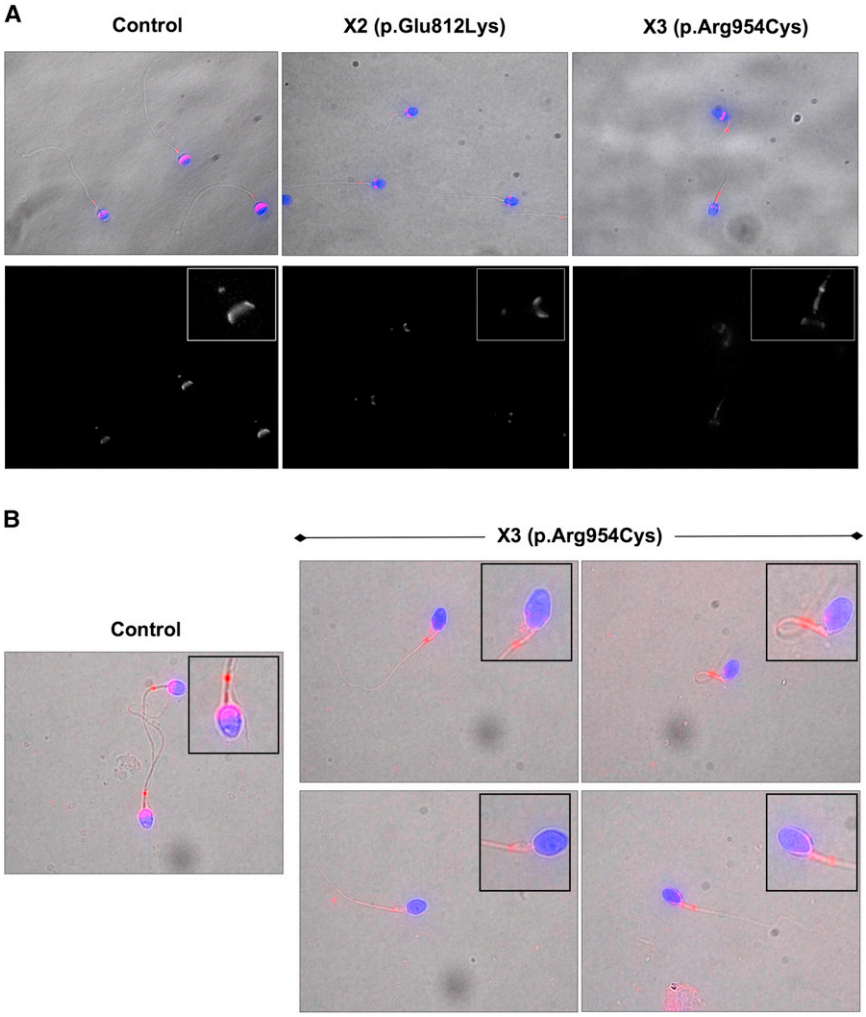

Figure 4. Immunodetection of SLC26A8 in Sperm from Individuals X2 and X3
Immunofluorescence studies were performed, as previously described,[24] with methanol- and acetone-fixed sperm preparations and a polyclonal antibody directed against SLC26A8 (SE5362). Samples were examined under a Nikon Eclipse 600 microscope, and digital images were acquired with NIS-Elements D (Nikon) software (identical instrument settings were used in each case) and analyzed with Image J 1.44cw software (National Institutes of Health).
(A) Upper panel: merged image with DAPI (blue), SLC26A8 (red), and phase contrast (gray). Lower panel: same image in dark field. The SLC26A8 signal is in white.
(B) Merged image with DAPI (blue), SLC26A8 (red), and phase contrast (gray).

SLC26 transporters appear to be general regulators, most often activators, of the CFTR channel, and their activity is essential for CFTR function in vivo. Further study of SLC26 transporters might improve not only the diagnosis and treatment of CF but also the quality of sperm for assisted reproduction procedures.

Supplemental Data

Supplemental Data include two figures and one table and can be found with this article online at http://www.cell.com/AJHG.

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Web Resources

The URLs for data presented herein are as follows:

1000 Genomes, http://browser.1000genomes.org/index.html
Online Mendelian Inheritance in Man (OMIM), http://omim.org/
PolyPhen, http://genetics.bwh.harvard.edu/pph/
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq

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